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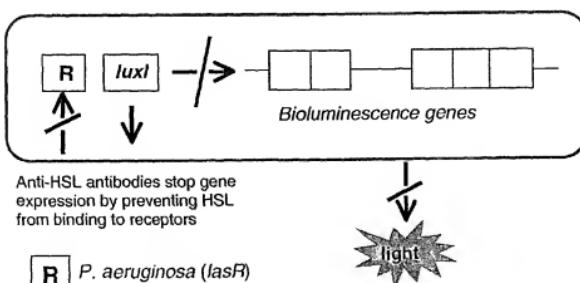
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(54) Title: METHODS FOR THE TREATMENT OF AN INFECTIOUS BACTERIAL DISEASE WITH AN ANTI-LACTONE OR LACTONE DERIVED SIGNAL MOLECULES ANTIBODY



(57) Abstract: The present invention relates to methods for the control of virulence of infectious bacteria by modulating the extra-cellular concentration of bacterial cell signalling molecules. Derivatives of cell signalling molecules are conjugated to suitable carrier proteins and used to isolate high affinity receptors recognising the native signal molecule(s). By binding to signalling molecules, the receptors reduce and maintain extra-cellular concentrations of signal molecules below the threshold level that would otherwise result in certain opportunistic pathogens adopting a virulent form, and can transform virulent organisms to non-virulent states. These receptors have applications for the treatment of individuals with susceptibility to infection, the treatment of patients with existing infections, in disease monitoring and management, and in related applications where the host for infection is an animal or plant.



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**METHODS FOR THE TREATMENT OF AN INFECTIOUS BACTERIAL DISEASE WITH AN ANTI-LACTONE OR LACTONE DERIVED SIGNAL MOLECULES ANTIBODY****Fields of the invention**

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The present invention relates to methods for controlling and treating bacterial infections in patients. The methods of the invention are applicable to most, if not all gram negative and gram-positive bacterial infections. The invention provides for the application of therapies based upon, in the preferred embodiment, immunoglobulin or 10 immunoglobulin-like receptor molecules that have affinity and specificity for signalling molecules involved in the processes of bacterial cell to cell communication. By binding to such molecules, the receptors can be used to diagnose the presence of bacteria or to assess the disease state of patients, and can further be used to control concentrations of molecules involved in inducing a virulent state in opportunistic and 15 other pathogens.

**Background of the invention**

One of the major causes of mortality and morbidity amongst patients undergoing 20 treatment in hospitals today is due to hospital acquired infection. Susceptibility to such infection can be as a result of the primary illness for which the patient was admitted, of immuno-suppressive treatment regimes, or as a consequence of injury resulting in serious skin damage, such as burns. The bacterium to which the highest proportion of cases is attributed is *Pseudomonas aeruginosa*. It is the epitome of an 25 opportunistic pathogen of humans. The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect, if the tissue defences are compromised in some manner. Although accounting for a relatively small number of species, it poses a serious threat to human health and is used hereafter as a representative example of an infectious bacterium, and does not in any 30 way limit the scope or extent of the present invention.

*Ps. aeruginosa* is an opportunistic pathogen that causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteraemia and a variety of systemic infections, particularly in victims of severe burns, and in cancer and AIDS patients who are immunosuppressed. Respiratory infections caused by *Ps. aeruginosa* occur almost exclusively in individuals with a compromised lower respiratory tract or a compromised systemic defence mechanism. Primary pneumonia occurs in patients with chronic lung disease and congestive heart failure. Bacteraemic pneumonia commonly occurs in neutropenic cancer patients undergoing chemotherapy. Lower respiratory tract colonisation of cystic fibrosis patients by mucoid strains of *Ps. aeruginosa* is common and difficult, if not impossible, to treat. It causes bacteraemia primarily in immuno-compromised patients. Predisposing conditions include haematologic malignancies, immuno-deficiency relating to AIDS, neutropenia, diabetes mellitus, and severe burns. Most *Pseudomonas* bacteraemia is acquired in hospitals and nursing homes where it accounts for about 25 percent of all hospital acquired gram-negative bacteraemias.

The bacterium is notorious for its natural resistance to many antibiotics due to the permeability barrier afforded by its outer membrane LPS and is, therefore, a particularly dangerous and dreaded pathogen. Also, its tendency to colonise surfaces in a biofilm form makes the cells impervious to therapeutic concentrations of antibiotics. Since its natural habitat is the soil, living in association with the bacilli, actinomycetes and moulds, it has developed resistance to a variety of their naturally occurring antibiotics. Moreover, *Pseudomonas* spp. maintain antibiotic resistance plasmids, both Resistance factors (R-factors) and Resistance Transfer Factors (RTFs), and are able to transfer these genes by means of the bacterial processes of transduction and conjugation. Only a few antibiotics are effective against *Pseudomonas*, including fluoroquinolone, gentamicin and imipenem, and even these antibiotics are not effective against all strains. Combinations of gentamicin and carbenicillin are reportedly effective in patients with acute *Ps. aeruginosa* infections. The futility of treating *Pseudomonas* infections with antibiotics is most dramatically illustrated in cystic fibrosis patients, virtually all of whom eventually become infected

with a strain that is so resistant it cannot be treated. Because of antibiotic resistance, susceptibility testing of clinical isolates is mandatory.

Ps. aeruginosa can usually be isolated from soil and water, as well as the surfaces of plants and animals. It is found throughout the world, wherever these habitats occur, so it is quite a "cosmopolitan" bacterium. It is sometimes present as part of the normal flora of humans, although the prevalence of colonisation of healthy individuals outside the hospital is relatively low (estimates range from 0 to 24 percent depending on the anatomical locale). In hospitals it is known to colonise food, sinks, taps, mops, respiratory equipment surgical instruments. Although colonisation usually precedes infections by Ps. aeruginosa, the exact source and mode of transmission of the pathogen are often unclear because of its ubiquitous presence in the environment. Amongst intensive care patients in whom infection is suspected on clinical grounds, as many as 50% have no identifiable source for infection. Currently 10 1,400 deaths worldwide are caused each day by Ps. aeruginosa in intensive care units (ICU's), making it the No 1 killer.

Ps. aeruginosa is primarily a nosocomial pathogen. According to the CDC, the overall incidence of Ps. aeruginosa infections in US hospitals averages about 0.4 percent (4 per 1000 discharges), and the bacterium is the fourth most commonly isolated nosocomial pathogen accounting for 10.1% of all hospital-acquired infections. Globally it is responsible for 16% of nosocomial pneumonia cases, 12% of acquired urinary tract infections, 8% of surgical wound infections and 10% of bloodstream infections. Immuno-compromised patients such as neutropenic cancer 20 and bone marrow transplant patients are susceptible to opportunistic Ps. aeruginosa infection, leading to 30% reported deaths. It is also responsible for 38% of ventilator-associated pneumonias and 50% of deaths amongst AIDS patients. In burns cases Ps. aeruginosa infections have declined in recent years due to improved treatment and dietary changes. Mortality rates however remain high, accounting for 60% all deaths 25 30 due to secondary infection of burns patients.

One reason for the versatility of *Ps. aeruginosa* is that it produces a diverse battery of virulence determinants including elastase, LasA protease, alkaline protease, rhamnolipids, type IV pilus-mediated twitching motility, pyoverdin (Williams et al., 1996, Stintzi et al., 1998, Glessner et al., 1999), pyocyanin (Brint & Ohman, 1995, Reimann et al., 1997) and the cytotoxic lectins PA-I and PA-II (Winzer et al., 2000). It is now known that many of these virulence determinants are regulated at the genetic level in a cell density-dependent manner through quorum sensing. *Ps. aeruginosa* possesses at least two quorum sensing systems, namely the *las* and *rhl* (*vsm*) systems which comprise of the LuxRI homologues LasRI (Gambello & Iglewski, 1991) and RhlRI (VsmRI) (Latifi et al., 1995) respectively (Figure 2). LasI directs the synthesis of 3-oxo-C12-HSL (Passador et al., 1993, Pearson et al., 1994) whereas RhII directs the synthesis of C4-HSL (Winson et al., 1995). The *las* and the *rhl* systems are thought to exist in a hierarchy where the *las* system exerts transcriptional control over RhlR (Williams et al., 1996, Pesci et al., 1997). The transcriptional activator LasR functions in conjunction with 3-oxo-C12-HSL to regulate the expression of the genes encoding for the virulence determinants elastase, LasA protease, alkaline protease and exotoxin A (Gambello & Iglewski, 1991, Toder et al., 1991, Gambello et al., 1993, Pearson et al., 1994) as well as *lasI*. Elastase is able to cleave collagen, IgG and IgA antibodies, complement, and facilitates bacterial adhesion onto lung mucosa. In combination with alkaline protease it also causes inactivation of gamma Interferon (INF) and Tumour Necrosis Factor (TNF). LasI directs the synthesis of 3-oxo-C12-HSL which together with LasR, binds to the *lasI* promoter and creates a positive feedback system. The RhlR transcriptional activator, along with its cognate AHL (C4-HSL), regulates the expression of *rhlAB* (rhamnolipid), *lasB*, *aprA*, *RpoS*, cyanide, pyocyanin and the lectins PA-I and PA-II (Ochsner et al., 1994, Brint & Ohman, 1995, Latifi et al., 1995, Pearson et al., 1995, Winson et al., 1995, Latifi et al., 1996, Winzer et al., 2000). These exist in a hierarchical manner where by the LasR/3-oxo-C12-HSL regulates *rhlR* (Latifi et al., 1996, Pesci et al., 1997) and consequently both systems are required for the regulation of all the above virulence determinants.

A number of different approaches are being actively pursued to develop therapeutics for the treatment or prevention of *Ps. aeruginosa* infection. Some are intended to be broad ranging while others are directed at specific types of *Pseudomonas* infection. Those that follow traditional routes include the development of vaccines such as that described in US patent 6,309,651, and a new antibiotic drug (SLIT) that is hoped will be effective against gram-negative bacteria in general but is designed primarily to act against *Ps. aeruginosa* and is administered by aerosol inhalation. A further observation under investigation is that the antibiotic erythromycin administered at sub-optimal growth inhibitory concentrations simultaneously suppresses the production of *Ps. aeruginosa* haemagglutinins, haemolysin, proteases and homoserine lactones (HSLs), and may be applicable for the treatment of persistent *Ps. aeruginosa* infection. Cream formulations containing amphipathic peptides are also being examined as a possible means of preventing infection of burns or other serious skin wounds. US patent 6,309,651 also teaches that antibodies against the PcrV virulence protein of *Ps. aeruginosa* may afford protection against infection.

There is also some interest in the modulation of homoserine lactone levels as a means of controlling pathogenicity. Certain algae have been demonstrated to produce competitive inhibitors of acyl-homoserine lactones (AHL's) such as furanones (Manefield, 1999), as have some terrestrial plants. These compounds displace the AHL signal molecule from its receptor protein and can act as agonist or antagonist in AHL bioassays (Tepletski et al., 2000). Other methods employed to reduce HSL concentration include the development of auto-inducer inactivation enzymes (AiiA's) that catalyse the degradation of HSLs.

There are a number of potential problems and limitations associated with the therapies currently under development. It is as yet unproven as to whether vaccines will be efficacious treatments. *Ps. aeruginosa* produces an extensive mucoid capsule that effectively protects against opsonisation by host antibodies, as revealed by patients with persistent infections having high serum titres of anti-*Pseudomonas* antibodies. A limitation in the applicability of treatments such as vaccines and anti-PcrV antibodies, as described in US patent 6,309,651, is that these approaches restrict themselves to

*Pseudomonas* infection, and would not be efficacious against other bacteria. The use of auto-inducer mimics are limited by the concentrations of most that are required to effectively compete against HSLs for the receptor binding site, and the possibility of side effects. It is well known that HSLs released by *Pseudomonas* and other bacteria have a number of direct effects on human physiology. These include inhibition of histamine release as described in WO 01/26650. WO 01/74801 describes that HSLs are also able to inhibit lymphocyte proliferation and down-regulate the secretion of TNF- $\alpha$  by monocytes and macrophages, so acting as a general immuno-suppressant. There is a danger therefore that therapies involving the use of competitive HSL mimics may result in down-regulation of the patient's immune system. This would be generally undesirable, and particularly so in immuno-compromised patients. The use of antibiotics can, at best, be viewed as a short-term strategy in view of the remarkable ability of this bacterium (and others) to develop resistance to antibiotics.

15 That the pathogenesis of *Ps. aeruginosa* is clearly multifactorial is underlined by the large number of virulence factors and the broad spectrum of diseases associated with this bacterium. Many of the extra-cellular virulence factors required for tissue invasion and dissemination are controlled by cell-to-cell signalling systems involving homoserine lactone-based signal molecules and specific transcriptional activator 20 proteins. These regulatory systems allow *Ps. aeruginosa* to adapt to a virulent form in a co-ordinated cell density dependent manner, and to overcome host defence mechanisms. Interference with such cell signalling and the associated production of virulence factors is a promising therapeutic approach to reducing illness and death caused by *Ps. aeruginosa*. The importance of such approaches is highlighted by the 25 growing number of bacterial pathogens found to utilise similar cell-to-cell signalling systems.

In order to study the molecular basis of host-pathogen interactions, it is desirable to have available a suitable model system (non-human) in which the stimuli and mechanisms relating to pathogenicity in humans can be replicated. In the case of many diseases the pathogen concerned is intrinsically associated with one, or a few 30 closely related species or groups, e.g. HIV. Other organisms can cause disease in a

wide range of host, crossing the species, genus, and even kingdom barriers. *Ps. aeruginosa* is one such pathogen, being able to infect a variety of both plant, insect and animal species.

- 5 In recent years it has been demonstrated that *Ps. aeruginosa* strains that are able to cause disease in humans and mice are also able to kill the nematode worm *Caenorhabditis elegans* (Tan *et al.*, 1999a, Tan *et al.*, 1999b, Tan *et al.*, 2000). More importantly, the pathogenicity of *Ps. aeruginosa* to *C. elegans* is regulated by the same cell density-dependant quorum sensing systems that control pathogenesis in  
10 humans. The recent completion of the sequencing of the genomes of both *Ps. aeruginosa* and *C. elegans* make this relationship ideal for the study of bacterial disease mechanisms. The fact that 36% of *C. elegans* proteins also have homologues in humans (Darby *et al.*, 1999), and the ease with which *C. elegans* can be grown in the laboratory, have lead to its widespread use as a model for pathogenesis and host  
15 defences in humans (Kurz and Ewbank, 2000).

A variety of different mechanisms by which *Ps. aeruginosa* mediates killing of *C. elegans* have been identified. Tan *et al.*, 1999a; 1999b, and Mahajan-Miklos *et al.*, 1999, describe the use of a clinical isolate (strain PA14) that also infects mice and  
20 plants. By varying the growth conditions of the bacteria, subsequent application to *C. elegans* can result in either fast killing (within hours) or slow killing (within 3 to 4 days. The fast killing mechanism is dependant only on the Rhl quorum sensing system. Moreover, the use of cell-free medium in which *Ps. aeruginosa* have been appropriately grown, or heat killed extracts are equally effective as death is effected  
25 by diffusible pyocyanin toxin. In contrast the slow killing mechanism is reliant on both Las and Rhl systems and results in significant infection of the nematode gut. As death is probably a result of infiltration of the host by the bacteria, this assay provides the most useful nematode model for infection in animals. A third killing mechanism has been described by Darby *et al.*, (1999). Here the use of *Ps. aeruginosa* strain  
30 PA01 (a known human pathogen) grown in brain-heart infusion medium results in

rapid paralysis and death of *C. elegans*. As with the slow killing described earlier, paralysis is both Las and Rhl system-dependant.

There is a need to develop effective means of modulating the concentrations of HSLs and other bacterial cell signalling molecules involved in pathogenicity by methods that do not have adverse side effects, and are unlikely to be evaded by pathogenic bacteria in the foreseeable future.

### Summary of the invention

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The present invention provides for methods for controlling the virulence of human, animal and plant pathogenic bacteria by regulating the extra-cellular concentrations of bacterial cell signalling molecules. Whereas other treatments are restricted to a particular pathogen or group of pathogens, or to specific aspects of bacterial virulence, the present invention addresses bacterial virulence in general.

According to a first aspect of the present invention, there is provided an antibody to a lactone or lactone-derived signal molecule secreted by bacteria.

20

Antibodies according to the present invention can be polyclonal antibodies or monoclonal antibodies. Polyclonal antibodies can be raised by stimulating their production in a suitable animal host (e.g. a mouse, rat, guinea pig, rabbit, sheep, chicken, goat or monkey) when the antigen is injected into the animal. If necessary an adjuvant may be administered together with the antigen. The antibodies can then be purified by virtue of their binding to antigen or as described further below. Monoclonal antibodies can be produced from hybridomas. These can be formed by fusing myeloma cells and B-lymphocyte cells which produce the desired antibody in order to form an immortal cell line. This is the well known Kohler & Milstein technique (*Nature* 256 52-55 (1975)).

25

Techniques for producing monoclonal and polyclonal antibodies which bind to a particular protein are now well developed in the art. They are discussed in standard

immunology textbooks, for example in Roitt *et al*, *Immunology* second edition (1989), Churchill Livingstone, London.

In addition to whole antibodies, the present invention includes derivatives thereof which  
5 are capable of binding to antigen. Thus the present invention includes antibody  
fragments and synthetic constructs. Examples of antibody fragments and synthetic  
constructs are given by Dougall *et al* in *Tibtech* 12 372-379 (September 1994).  
Antibody fragments include, for example, Fab, F(ab')<sub>2</sub> and Fv fragments (see Roitt *et al*  
[supra]). Fv fragments can be modified to produce a synthetic construct known as a  
10 single chain Fv (scFv) molecule. This includes a peptide linker covalently joining V<sub>H</sub>  
and V<sub>L</sub> regions which contribute to the stability of the molecule. The present invention  
therefore also extends to single chain antibodies or scAbs.

Other synthetic constructs include CDR peptides. These are synthetic peptides  
15 comprising antigen binding determinants. Peptide mimetics may also be used. These  
molecules are usually conformationally restricted organic rings which mimic the  
structure of a CDR loop and which include antigen-interactive side chains. Synthetic  
constructs also include chimaeric molecules. Thus, for example, humanised (or  
20 primatised) antibodies or derivatives thereof are within the scope of the present  
invention. An example of a humanised antibody is an antibody having human  
framework regions, but rodent hypervariable regions. Synthetic constructs also include  
molecules comprising a covalently linked moiety which provides the molecule with  
some desirable property in addition to antigen binding. For example the moiety may be  
25 a label (e.g. a detectable label, such as a fluorescent or radioactive label) or a  
pharmaceutically active agent.

In order to generate anti-bacterial signal molecule antibodies, it is preferable to  
conjugate the target molecule, or a suitable derivative, to two different carrier  
molecules (proteins), though a single conjugated species can be also used. Bacterial  
30 signal molecules, in general, are too small to stimulate an immune response *in-vivo*, or  
to be used directly as a source of antigen for the selection of high affinity antibodies  
from antibody libraries. Selection of antibodies specific for the cell signalling

molecule (hereafter referred to as 'antigen') is carried out in the preferred embodiment using a repertoire (library) of first members of specific binding pairs (sbp), for example a library of antibodies displayed on the surface of filamentous bacteriophage. Any other system that allows for the selection of specific receptors from a library of receptors is also applicable for the methods of the present invention.

In alternative embodiments signal molecule-specific clones can be selected from a panel of antibody secreting hybridoma cell lines generated from an animal immunised with an antigen conjugate. For the purposes of a general illustration the example of a library of antibody binding sites displayed on phage particles will be used.

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A conjugate comprising an antigen coupled to a suitable carrier molecule, which can be a protein, a peptide or any natural or synthetic compound or material (referred to hereafter as 'conjugate-1') is immobilised onto a suitable solid support such as an 'immunotube' or microtitre plate, and the uncoated surface blocked with a non-specific blocking agent such as dried milk powder. Suitable conjugate molecules can include, but are not limited to proteins such as bovine serum albumin (BSA), Keyhole Limpet Haemocyanin (KLH), Bovine Thryroglobulin (TG), Ovalbumin (Ova), or non-proteins such as biotin. The only restriction on the selection of the conjugate molecule is that it be immobilisable in some way and for immunisation is large enough to elicit an immune response.

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A library of first members of specific binding pairs (sbp's) ('the library') is applied to the immobilised conjugate and incubated for sufficient time for sbp members recognising conjugate-1 to bind. Phage not recognising the conjugate are removed by stringent washing. Phage that remain bound are eluted, for example with triethylamine or other suitable reagent, into a buffer solution to restore neutral pH. Recovered phage particles are then infected into a suitable host organism, e.g. *E. coli* bacteria, and cultured to amplify numbers of each selected member and so generate a second 'enriched' library. The process is then repeated using the enriched library to select for phage-antibodies ('phage') recognising the antigen conjugated to a second carrier protein (conjugate-2).

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Additional rounds are performed as required, the selection process being altered to favour selection of those sbp members recognising the free form of the antigen. Phage are selected against antigen conjugates as described previously, using initially conjugate-1, and alternating with conjugate-2 (where available) for each subsequent  
5 round. Bound phage are eluted by incubating with a solution of free antigen, or antigen conjugated to small soluble selectable moieties, e.g. biotin, for sufficient time for sbp members with higher affinity for the bound form of the antigen to dissociate from the immobilised conjugate. Those phage eluted with free antigen are infected into *E. coli* cells for amplification and re-selection, and those remaining bound to the  
10 immobilised antigen discarded. Alternatively, but less preferably, all antibodies binding to conjugate may be eluted eg. with low pH.

Individual (monoclonal) phage clones from each round of selection are screened for desired binding characteristics. This can be performed by a variety of methods that  
15 will be familiar to those with ordinary skill in the art, depending on requirements, including such techniques as SPR (Surface Plasmon Resonance) and ELISA (Enzyme Linked Immuno-Sorbant Assay). Selection criteria will include the ability to bind preferentially to the free soluble form of the antigen in the presence of conjugated derivatives.  
20

In the preferred embodiment of the invention, antibodies will be generated from a naïve human antibody phage display library (McCafferty et al., Nature 348: 552-554, 1990; and as described in WO 92/01047). Thus the antibodies could be used for administering to patients in addition to use as diagnostic or dialysis reagents. In a  
25 diagnostic assay the antibody could be used to determine the presence and concentration of HSLs in patients and so predict the patient's infection status. In other embodiments a library can be constructed from an animal pre-immunised with one or more conjugates of a HSL and a suitable carrier molecule. A further alternative is the generation of hybridoma cell lines from an animal immunised as  
30 described above. In the latter two cases it is preferable that steps be taken to reduce the immunogenicity of resulting antibodies, for example by creating host animal-human chimaeric antibodies, or "humanisation" by CDR grafting onto a suitable

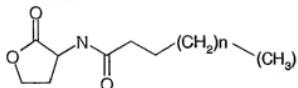
antibody framework scaffold. Other methods applicable will include the identification of potential T-cell epitopes within the antibody, and the subsequent removal of these e.g. by site-directed mutagenesis (de-immunisation). In a further embodiment the antibody can be engineered to include constant regions from different classes of human immunoglobulin (IgG, IgA, etc.) and produced as a whole antibody molecule in animal cells. In particular these approaches are desirable where the antibodies are to be used therapeutically

- 5 For the present invention, the antibody may be monoclonal or polyclonal. The antibodies may be human or humanised, or for dialysis / diagnostic applications may be from other species. Antibody fragments or derivatives, such as Fab, F(ab').sup.2 (also written as F(ab')<sub>2</sub>), Fv, or scFv, may be used, as may single-chain antibodies (scAb) such as described by Huston et al. (Int. Rev. Immunol. 10: 195-217, 1993), domain antibodies (dAbs), for example a single domain antibody, or antibody-like 10 single domain antigen-binding receptors. In addition to antibodies, antibody fragments and immunoglobulin-like molecules, peptidomimetics or non-peptide mimetics can be designed to mimic the binding activity of antibodies in preventing or modulating bacterial infection by inhibiting the binding of cell-signalling molecules.
- 15 20 After the preparation of a suitable antibody, it may be isolated or purified by one of several techniques commonly available (for example, as described in *Antibodies: A Laboratory Manual*, Harlow and Lane, eds. Cold Spring Harbor Laboratory Press (1988)). Generally suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on Protein A or Protein G columns, or combinations 25 of these techniques. Recombinant antibodies can be prepared according to standard methods, and assayed for specificity using procedures generally available, including ELISA, ABC, dot-blot assays etc.

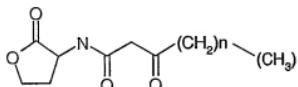
30 The lactone signal molecule may be a homoserine molecule, or a peptide thiolactone molecule.

The homoserine lactone molecule can have a general formula selected from the group

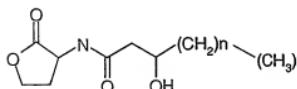
consisting of:



Formula I



Formula II



Formula III

where n = 0 to 12.

5

Compounds of general formula I can be described as acyl-homoserine lactone molecules. Compounds of general formula II can be described as 3-oxo-homoserine lactones. Compounds of general formula III can be described as 3-hydroxy-homoserine lactones.

10

Preferred homoserine lactone molecules for general formula I are *N*-butanoyl-L-homoserine lactone (BHL) where n = 0, *N*-dodecanoyl-L-homoserine lactone (dDHL) where n = 8 and *N*-tetradecanoyl-L-homoserine lactone (tDHL) where n = 10.

15

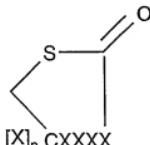
Preferred homoserine lactone molecules for general formula II are *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) where n = 2 and *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) where n = 8. Preferred homoserine lactone molecules for general formula III are *N*-(3-hydroxybutanoyl)-L-homoserine lactone (HBHL) where n = 0.

20

In general the bacterial HSLs can be further subdivided into two classes: i) long chain molecules (10-12 carbons) and ii) short chain molecules (4-8 carbons). In *Pseudomonas* sp these different size classes bind to different R molecules and cause

different genes to be switched on. Long chain molecules bind to the R homologue gene product known as LAS and short chain molecules to the RHL protein homologue.

- 5 The peptide thiolactone can have a general formula (IV) as follows:



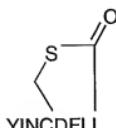
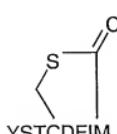
where X is any amino acid and n = 1 to 10.

- In the above, and throughout this specification, the amino acid residues are designated  
10 by the usual IUPAC single letter nomenclature. The single letter designations may be correlated with the classical three letter designations of amino acid residues as follows:

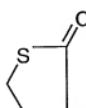
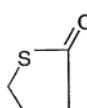
	A = Ala	G = Gly	M = Met	S = Ser
	C = Cys	H = His	N = Asn	T = Thr
15	D = Asp	I = Ile	P = Pro	V = Val
	E = Glu	K = Lys	Q = Gln	W = Trp
	F = Phe	L = Leu	R = Arg	Y = Tyr

Preferred peptide thiolactone molecules may have the following structures:

20



30

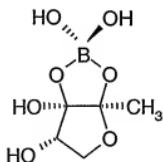


A growing number of bacterial species are being found to communicate between cells  
5 using a variety of small signal molecules. Gram-negative bacteria predominantly use  
N-acyl homoserine lactones (Table 1). The latter are a group of compounds that share  
a common homoserine lactone ring structure and vary in the length and structure of a  
side chain (Figure 1a). There are three classes within the group, the acyl-homoserine  
lactones, the 3-oxo-homoserine lactones and the 3-hydroxy-homoserine lactones. A  
10 single species can produce and respond to members of more than one class.

The lactone-derived signal molecule may be a furanosyl borate diester, for example,  
AutoInducer-2 or AI-2, Pro-AI-2 or a Pro-AI-2-reactive hapten (Figure 1b). Many  
gram negative and gram positive organisms such as *Vibrio harveyi* and *Bacillus*  
15 *anthracis* produce a second signal molecule, AI-2, that is derived from the same S-  
Adenosylmethionine source as homoserine lactones, and binds to the receptor LuxP  
(Figure 1b). It is thought likely that AI-2 is a universal bacterial signal molecule,  
being produced and recognised by and induces virulence in a wide variety of species.

20

Auto Inducer-2 (AI-2)



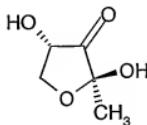
30

AI-2 can be described as 2,3-dihydroxy-4-methyl-3,4-borate diester.

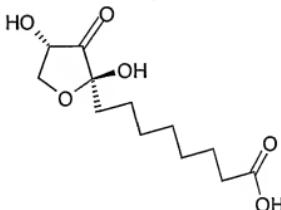
A lactone-derived signal molecule can also be a derivative of 4,5-dihydroxy-2,3-  
35 pentanedione (DPD) which cyclizes naturally to form Pro-AI-2, which reacts  
naturally with boric acid to form AI-2 (Figure 1b). Pro-AI-2 can be described as 2,4-  
dihydroxy-4-methyl-furan-3-one. Pro-AI-2 can be derivatised as shown in Figure 1b  
at the 4-methyl position to add a heptanoic acid moiety to form a Pro-AI-2 reactive

hapten. Other derivatives may also include other straight chain or branched, saturated or unsaturated C<sub>1</sub>-C<sub>10</sub> carboxylic acid moieties, such as methanoic, ethanoic, propanoic, pentanoic, hexanoic, heptanoic, octanoic, nonanoic or decanoic acid.

10 Pro AI-2



20 Pro AI-2 reactive hapten



30 Gram-positive bacteria such as *Staphylococcus aureus* use short peptides (Figure 1c) (Mayville *et al.*, 1999). The cells use the molecules as a means of determining the local cell density, such that in conditions of low cell density the concentration of signal molecule is correspondingly low. In high cell densities the local signal molecule concentration is high. When this concentration reaches a threshold level it induces the transcription of genes involved in virulence and the onset of a disease state in the host.

35

40 The thiolactone derivatised peptide signal molecules used by *Staphylococcus* spp. have additional biological functions. They not only provide the bacteria with information about their local population density, but they also serve to suppress virulence in other *S. aureus* belonging to different sub-groups (Lyon *et. al.*, 2000). This bi-functionality is split between the different structural elements of the peptide, with the thiolactone C-terminus inhibiting virulence in other sub-groups. The un-

modified N-terminus acts as the signal to up-regulate virulence gene expression in the sub-group that synthesised it, but only in conjunction with the C-terminus, which is also required. The presence of a truncated peptide comprising the C-terminal 5 amino acids with thiolactone linkage suppresses not only the other three sub-groups, but also  
5 the strain that produced it. Thus it follows that an antibody that recognises the N-terminus of the signal peptide, and effectively displays the C-terminus by leaving it exposed, will effectively suppress virulence in all *S. aureus* strains. Antibodies of the present invention may therefore be raised against an epitope presented by the thiolactone molecule as described above or a structural element thereof, for example  
10 the peptide sequence or the thiolactone moiety.

In certain preferred embodiments of the invention, the antibodies are scAbs, in particular scAbs that are obtained from *E. coli* clones designated as XL1-Blue G3H5, G3B12, G3G2 and/or G3H3. The clones have been deposited at NCIMB, Aberdeen,  
15 UK on 18 March 2003 under the terms of The Budapest Treaty under the following accession numbers: G3H5 deposited as NCIMB-41167, G3B12 deposited as NCIMB-41168, G3G2 deposited as NCIMB-41169 and G3H3 deposited as NCIMB-41170. The strains may be cultivated in an appropriate growth media such as LB media supplemented with 100 $\mu$ g/ml ampicillin, optionally supplemented with 12.5 $\mu$ g/ml  
20 tetracycline, and/or 1% glucose, under standard conditions of 37°C in air.

Bacterial signalling molecules are being discovered in every organism for which they are searched. It seems to be a ubiquitous system, applicable to every species. The main differences are that all gram negative (gram -ve) bacteria use homoserine  
25 lactone-based molecules, and gram positive (gram +ve) bacteria use (modified) small peptides. Many gram negative and gram positive organisms such as *Vibrio harveyi* and *Basillus anthracis* (Jones, M.B. and Blaser, M.J.) also use a small boron-containing organic molecule AI-2 (AutoInducer-2) which, like homoserine lactones, is derived from S-Adenosylmethionine. Previous work in this field has concentrated  
30 on mimicking signal molecules with ones that are recognised but that do not function, i.e. no pathogenic switching, or on blocking the various receptor systems. The disadvantages of these methods are principally that resistance can be developed to the

mimic or block and the 'real' signal molecule is still there and will compete for binding. In addition, some bacterial signalling molecules e.g. homoserine lactones are virulence factors in their own right, and can directly cause immuno-suppression of the host (i.e. patient). The essence of the present invention is to target the actual signal molecule, and this can be applied to all bacterial cell-to-cell signalling systems (gram negative and gram positive). This approach has a key and important advantage over all previous efforts in the field in that the bacteria will not recognise that they are being attacked, they will simply detect that they are alone. There will not be any selective pressure for resistance.

10

According to a second aspect of the present invention, there is provided a pharmaceutical composition comprising an antibody as defined in the first aspect of the invention.

15

Such compositions may be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with a carrier(s), diluent (s) or excipient(s) under sterile conditions.

20

The pharmaceutical composition may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) route. Such compositions may be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with the carrier(s) or excipient(s) under sterile conditions.

25

Pharmaceutical compositions adapted for oral administration may be presented as discrete units such as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids; or as edible foams or whips; or as emulsions)

30

Suitable excipients for tablets or hard gelatine capsules include lactose, maize starch or derivatives thereof, stearic acid or salts thereof.

Suitable excipients for use with soft gelatine capsules include for example vegetable oils, waxes, fats, semi-solid, or liquid polyols etc.

5 For the preparation of solutions and syrups, excipients which may be used include for example water, polyols and sugars. For the preparation of suspensions oils (e.g. vegetable oils) may be used to provide oil-in-water or water in oil suspensions.

10 Pharmaceutical compositions adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis as generally described in *Pharmaceutical Research*, 3 (6), page 318 (1986).

15 Pharmaceutical compositions adapted for topical administration may be formulated as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. For infections of the eye or other external tissues, for example mouth and skin, the compositions are preferably applied as a topical ointment or cream. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water cream base or a water-in-oil base. 20 Pharmaceutical compositions adapted for topical administration to the eye include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent. Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouth washes.

25 Pharmaceutical compositions adapted for rectal administration may be presented as suppositories or enemas.

30 Pharmaceutical compositions adapted for nasal administration wherein the carrier is a solid include a coarse powder having a particle size for example in the range 20 to 500 microns which is administered in the manner in which snuff is taken, i.e. by rapid

inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable compositions wherein the carrier is a liquid, for administration as a nasal spray or as nasal drops, include aqueous or oil solutions of the active ingredient.

5 Pharmaceutical compositions adapted for administration by inhalation include fine particle dusts or mists which may be generated by means of various types of metered dose pressurised aerosols, nebulizers or insufflators.

10 Pharmaceutical compositions adapted for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

15 Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injection solution which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation substantially isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. Excipients which may be used for injectable solutions include water, alcohols, polyols, glycerine and vegetable oils, for example. The compositions may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) 20 condition requiring only the addition of the sterile liquid carried, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

25 The pharmaceutical compositions may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts (substances of the present invention may themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to the substance of the present invention.

30 Dosages of the pharmaceutical compositions of the present invention can vary between wide limits, depending upon the disease or disorder to be treated, the age and condition

of the individual to be treated, etc. and a physician will ultimately determine appropriate dosages to be used.

Such compositions may be formulated for human or for veterinary medicine. The  
5 present application should be interpreted as applying equally to humans as well as to  
animals, unless the context clearly implies otherwise.

According to a third aspect of the invention, there is provided a method for the  
treatment of bacterial infection of a subject, the method comprising administration of  
10 an antibody of the first aspect of the invention to the subject.

Examples of bacteria found to cause disease states are shown in Table 1. Methods of  
this aspect of the invention therefore extend to a method of treatment of an infection by a  
strain of bacteria as shown in Table 1 in a subject. In a preferred embodiment of the  
15 invention, there is provided a method of treatment of an infection of *Pseudomonas*  
*aeruginosa* in a subject.

Therapeutic substances of the present invention may be used in the treatment of a human  
or non-human animal. The treatment may be prophylactic or may be in respect of an  
20 existing condition.

The antibody will usually be supplied as part of a sterile, pharmaceutical composition  
which will normally include a pharmaceutically acceptable carrier. This pharmaceutical  
composition may be in any suitable form, (depending upon the desired method of  
25 administering it to a patient).

It may be provided in unit dosage form, will generally be provided in a sealed container  
and may be provided as part of a kit. Such a kit of parts would normally (although not  
necessarily) include instructions for use. It may include a plurality of said unit dosage  
30 forms.

The methods of the invention can be applied to short or long-term, acute or chronic illness/disease, and is effective against most or all bacterial pathogens of plants, animals, including humans. The invention can also be used as a prophylactic treatment for the prevention of disease onset in individuals at risk of or from exposure 5 to pathogenic bacteria. The invention also has the potential to limit or prevent the down-regulation of the immune system that results from many infections, and is of particular concern with patients suffering from cancer, cystic fibrosis, AIDS and other immuno-suppressive conditions. Furthermore, as the methods of the invention are directed particularly at bacterial cell signalling molecules, and not primarily at the 10 bacterial cells themselves, there will be no selective pressure exerted on bacterial populations to develop resistance to the treatments described.

15 The antibody may be administered to infected patients in order to modulate and reduce bacterial infection. This can include inhalation of the antibody in an aerosol by cystic fibrosis patients to increase life expectancy.

In yet another embodiment the antibody is administered to immuno-suppressed patients in order to increase immuno-competence.

20 In yet another embodiment conjugates of cell signalling molecules to immunogenic proteins can be administered to individuals or patients in order to stimulate an immune response against the signalling molecule resulting in the generation of neutralising antibodies.

25 In yet another embodiment the antibody is used as an immuno-diagnostic reagent to detect the presence of, and/or pathogenic status of potential pathogens, for example in the bloodstream or pleural fluids of patients.

30 In yet another embodiment the antibody is used as an immuno-capture reagent to selectively remove bacterial cell signalling molecules from patient's blood in a form of dialysis.

In yet another embodiment alternative methods can be applied to the removal of bacterial cell-cell signalling molecules from the blood of a patient with a view to modulating the pathogenicity and virulence of infecting micro-organisms. This can be achieved with other natural receptors or molecules based on natural receptors that bind to said signal molecules. Alternatively non-natural receptors can be applied such as molecularly imprinted polymers (MIPs). This class of receptor have already been shown to be able to bind specifically to small molecular weight bio-molecules such as drugs (Hart et al., 2000) and steroids (Whitcombe et al., 1995; Ramstrom et al., 1996; Rachkov et al., 2000). In a further alternative dialysis can be achieved by the non-specific removal of all small molecular weight molecules from the patient's blood as is kidney dialysis.

In yet another embodiment the receptor may have catalytic or enzymatic activity, and be able to convert the cell signalling molecule into a form that is no longer recognised by the target organism, or no longer results in pathogenic switching.

In yet another embodiment the antibody is used in one or more of the above applications in combination, or in combination with other therapies, for example antibiotics, to provide additive and enhanced therapeutic regimes, disease monitoring and treatment management.

The antibodies (or equivalent) of the present invention could be administered to treat bacterial infection, or used as a preventative measure for those at high risk of infection. In the case where infection already exists, the antibodies may be administered alone or in combination with anti-bacterial antibodies or antibiotics or other anti-microbial treatments. Administration of anti-HSL antibodies in conjunction with other therapies may allow the use of shorter courses or lower doses of therapeutics, so decreasing the risk of resistance arising and improving patient compliance.

30

According to a fourth aspect of the invention there is provided an antibody as defined in the first aspect for use in medicine.

According to a fifth aspect of the invention, there is provided the use of an antibody as defined in the first aspect in the preparation of a medicament for the treatment of bacterial infection.

5

According to a sixth aspect of the invention, there is provided a method of screening a population of specific binding molecules for an anti-bacterial specific binding molecule, the method comprising conjugating a bacterial lactone signal molecule to a carrier molecule and using the conjugate so formed to identify a specific binding molecule that specifically binds to the conjugate from the population of specific binding molecules.

10

Such methods are therefore a means for identifying a specific binding molecule that can be used as an anti-bacterial agent, for example in the treatment of a bacterial infection. The specific binding molecule is an antibody or a fragment thereof, for example a monoclonal antibody, or a polyclonal antibody. Suitably the carrier molecule is a protein as described above. The population of specific binding molecules may be a phage display library.

15

20 Specific binding molecules identified by a method of the present invention may be used in medicine or a method of treatment as described above. The specific binding molecules may further be used in the preparation of a medicament for the treatment of a bacterial infection.

25

Such methods therefore extend to uses of a bacterial lactone signal molecule to screen a population of specific binding molecules in order to identify a specific binding molecule that specifically binds to said bacterial lactone signal molecule.

30

According to a seventh aspect of the invention, there is provided a method of treatment of a bacterial infection of a subject, the method comprising isolation of a bacterial lactone signal molecule in a sample from said subject and using said bacterial lactone signal molecule to screen a population of specific binding molecules

for an anti-bacterial specific binding molecule to identify a specific binding molecule that specifically binds to the signal molecule, and administering said specific binding molecule so identified to a patient in need thereof.

- 5 Such methods permit the identification of specific binding molecules directed against the infecting bacterial organisms whose signalling molecules are found in the sample. The sample may be of blood, saliva, tissue, cerebro-spinal fluid, tears, semen, urine, faeces, pus, skin, or mucous secretions. Samples of blood may be of whole blood, or of fractionated blood, for example, blood plasma. Tissue samples may be a biopsy of  
10 any infected or potentially infected tissue or organ. Samples may also be taken from wounds or sites of injury or infection or potential infection. Samples of fluid from the lungs or the contents of the stomach or the intestines may also be used.

15 Preferred features for the second and subsequent aspects of the invention are as for the first aspect *mutatis mutandis*.

Other objects, features and advantages of the present invention, including but not limited to related applications in plant and animal hosts, will be apparent to those skilled in the art after review of the specification and claims of the invention.

- 20 It will be apparent to those of ordinary skill in the art that the compositions and methods disclosed herein may have application across a wide range of organisms in inhibiting, modulating, treating or diagnosing disease or conditions resulting from infection. The compositions and methods of the present invention are described with reference to *Pseudomonas aeruginosa*, but it is within the competence of one of  
25 ordinary skill in the art to apply the objects herein to other species.

The invention will now be further described by reference to the non-limiting example and figures detailed below.

30

#### Description of Figures

Table 1 lists various bacterial phenotypes, with the cell signalling molecules and regulatory elements of the quorum sensing system that regulate them, for a range of organisms.

5 Table 2 shows a summary of the sensitivities (IC<sub>50</sub>) of anti-AHL scAbs to free antigen (dDHL-COOH) and to two AHL analogues (tDHL and OHHL) in competition with dDHL-BSA as determined by competitive inhibition ELISA.

10 Table 3 shows a comparison of the kinetics of two anti-AHL scAbs binding to immobilised dDHL-BSA conjugate as determined by Surface Plasmon Resonance using a BIAcore 2000 instrument. The association constants (ka), dissociation constants (kd) and affinity constants (KA, KD) are given.

15 Table 4 shows a summary of the sensitivities (IC<sub>50</sub>) of anti-HSL clones derived from chain-shuffling to various HSLs. Enclosed in brackets ( ) below each new clone is the designation of the clone from which it was derived. The degree of increased sensitivity to antigen of new clones over the starting clone is given in brackets ( ) where applicable. Data compare the binding to free HSLs in competition with dDHL-TG conjugate as determined by competition ELISA.

20 Table 5 shows the effects of anti-HSL scAbs in reducing the expression of the virulence factor elastase by *Ps. aeruginosa*. Data represent the ratio of clearance zone to colony area, expressed as a percentage compared to the PBS control (100%).

25 Figure 1(a) shows the chemical structures of the three representative classes of homoserine lactone bacterial cell signalling molecules. These differ in the substitution at position C3, and vary within each class by the length of the acyl side-chain (typically n=0 to n=10). In addition, there may be a cis-bond present within the acyl chain. Figure 1(b) shows the structures of i) pro AI-2, the immediate precursor of AI-2, ii) the boron-containing active AI-2 molecule and iii) the reactive pro-AI-2 hapten used to make conjugates; and Figure 1 (c) shows examples of thiolactone

peptide signalling molecules used by i) *Staphylococcus aureus* Group I, ii) *S. aureus* Group II, iii) *S. aureus* Group III and iv) *S. aureus* Group IV.

Figure 2 illustrates the genetic regulation of quorum or cell density dependent sensing. The cell signalling mechanism consists of two components: 1) the *I* gene (*lasI* and *rhlI*) homologues synthesise increasing quantities of bacterial cell signalling molecules (HSLs) throughout growth (hence quorum sensing), and 2) the concentration dependant binding of signalling molecules to a cognate R protein homologue (encoded by *lasR* and *rhlR*) which in turn can switch on a series of particular genes (operon), allowing bacteria to co-ordinate a density dependent phenotypic switch (eg. virulence, swarming).

Figure 3 illustrates the principals of the bioluminescent reporter gene assay using plasmid pSB1075. A reduction in light output is indicative of the successful blocking of bacterial cell signalling.

Figure 4 shows a comparison of the abilities of HSL-specific and irrelevant single-chain antibodies immobilised onto an inert column matrix to remove HSL from solution by immuno-affinity capture. Column eluates were applied to an *E. coli* surrogate of *Vibrio fischeri* (JM107-pSB401) and the effect of residual HSL in the eluates determined from the subsequent stimulation of the bacterial cultures to fluoresce as measured by RLU. ScAbs G3B12 and G3G2 are HSL-specific, anti-VZV is specific for a viral protein, anti-Paraquat and anti-Atrazine are specific for herbicides with molecular weights similar to HSL molecules, and the Resin control contained no immobilised scAb. Data represents the means of three replicate samples from two separate assays. Standard errors are indicated.

Figure 5 shows the inhibitory effects of specific and irrelevant single-chain antibodies on the dDHL-mediated stimulation of an *E. coli* surrogate of *P. aeruginosa* (JM109-pSB1075) as measured by bioluminescence output. Data is given for the HSL-specific scAb G3H5 (●), a non-specific control scAb (▼) (specific for a pathogenic

bacterial surface protein), and in the absence of scAb (○). Data points represent the means of three replicate samples from replicate assays.

Figure 6 shows the inhibitory effects of specific and irrelevant single-chain antibodies on the tDHL-mediated stimulation of an *E. coli* surrogate of *Ps. aeruginosa* (JM109-pSB1075) as measured by bioluminescence output. Data is given for three HSL-specific scAbs; G3H3 (●), G3G2 (■) and G3B12 (□), for the irrelevant anti- V scAb (○) (specific for a pathogenic bacterial surface protein), and in the absence of scAb (▽). Data points represent the means of three replicate samples from replicate assays.

10

Figure 7 shows the inhibitory effects of specific and irrelevant (non-specific) single-chain antibodies on the BHL-mediated stimulation of an *E. coli* surrogate (JM109-pSB406) of *Ps. aeruginosa Rrhl* system (short-chain HSL responsive) as measured by bioluminescence output after (a) 60 min and (b) 150 min. Data is presented for G3H5, G3B12 and G3H3 antibodies, a non-specific control antibody (specific for a pathogenic bacterial surface protein), and in the absence of antibody (PBS buffer only). Data points represent the means of three replicate samples from replicate assays.

15

Figure 8 shows the slow kill nematode assay demonstrating the ability of G3H5 and G3B12 antibodies to protect nematodes against infection by (a) the bacterial pathogen *Pseudomonas aeruginosa* strain PA14 and (b) *Ps. aeruginosa* strain PA01.

20

Figure 9 shows competition ELISA data for anti-peptide scAb YST-1 binding to BSA control, or BSA-peptide conjugate in the presence or absence of free peptide 'YSTGGAGSGG' or free thiolactone peptide Agr-D1 (see Figure 1c).

### Example 1

30

The examples described herein relate to *Vibrio fisheri* and *Pseudomonas aeruginosa*. These are given only as an example, the scope of the invention not being limited to the example but including all bacterial cell-to-cell signalling molecules that directly or

indirectly regulate expression of genes involved in virulence or pathogenicity, and also including other signal molecule-induced phenotypic changes to bacterial cells such as but not limited to bioluminescence.

5 A derivative of a HSL was synthesised (designated dDHL-COOH), having a twelve-carbon acyl chain acting as a 'linker', and terminating in a carboxylic acid group (see Figure 1). This was conjugated, via the carboxylic acid group, to the carrier proteins Bovine Serum Albumin (BSA) and Keyhole Limpet Haemocyanin (KLH) to produce dDHL-BSA and dDHL-KLH. Briefly, 50 mg BSA or KLH was dissolved in 1.67 ml  
10 water, and to it added 3.3 ml of 2 mM KH<sub>2</sub>PO<sub>4</sub> at pH 8.5, all at 4°C. To this, 1.05 ml dry dimethylformamide (DMF) was added drop-wise while stirring. Ten milligrams of activated *N*-hydroxysuccinimide ester of dDHL-COOH was dissolved in 100 µl dry DMF, and again added slowly to the carrier protein solution at 4°C. The reaction mixture was stirred well and allowed to stand for 24 h at 4°C. The conjugated material was then dialysed against 4 × 1 litre water, and conjugation confirmed by  
15 MALDI-TOF mass spectroscopy.

The term 'linker' refers to any chemical group used to allow attachment of the hapten (antigen) to a (preferably) immunogenic carrier molecule such that the hapten is displayed away from the surface of the carrier.  
20

In alternative objects of the invention, other carrier molecules such as magnetic beads or biotin, and other linkers and conjugation strategies can be employed. The two conjugated forms of dDHL were then used to screen an antibody phage display library. Briefly, the library was screened for a total of 3 rounds of bio-panning. In each round a dDHL-conjugate was immobilised onto a solid support and incubated with the library of phage-antibodies for sufficient time for phage-antibodies recognising the conjugate to bind. Unbound phage were removed by stringent washing with PBS (Phosphate Buffered Saline) and PBS-Tween, and the remaining bound phage eluted by incubation at low pH (round 1). Eluted phage were then infected into *E. coli* bacteria and amplified by methods familiar to those practised in  
25  
30

the art. The resulting amplified library of enriched clones was then used for the following round of panning. In order to reduce the numbers of clones selected that recognised the carrier protein, the immobilised conjugate (dDHL-BSA or dDHL-KLH) was alternated with successive rounds of selection. In order to bias selection in favour of clones recognising a specific HSL, the chosen HSL (dDHL-COOH) was used to competitively elute phage-antibodies during rounds 2 and 3, rather than low pH. Individual phage clones from round 3 were screened by ELISA: Each clone was assayed initially for the ability to bind to each of the dDHL-conjugates and to the carrier proteins alone. Those clones able to bind both conjugates but unable to bind either carrier protein were further assayed to identify those whose binding to conjugate could be inhibited by the presence of free dDHL-COOH in solution. The antibody variable region genes from those phage clones found to bind to free dDHL-COOH were sub-cloned into a soluble expression vector (pIMS 147), and produced as soluble single-chain antibody fragments (scAb) comprising the variable heavy and light chain domains joined by a flexible peptide linker, and a kappa constant domain from a human antibody. Quantification of the binding of soluble scAb to free HSLs was determined by competitive inhibition ELISA. Samples containing a constant concentration of each selected scAb (with respect to 1 microgram per ml dDHL-BSA) were incubated with a range of concentrations of free dDHL-COOH (or dDHL-conjugate) for 1 h, then applied to an ELISA plate coated with dDHL-BSA. After 1 h incubation, unbound scAb was washed off and any scAb remaining bound to the immobilised conjugate detected with enzyme-labelled anti-human kappa antibody. The sensitivity of scAb for free dDHL-COOH, and cross reactivity with other HSLs (tDHL and OHHL) was determined from the concentration of free antigen that reduced the binding of scAb (without free antigen) to dDHL-BSA by 50% (IC<sub>50</sub>) (Table 2).

The binding kinetics for anti-HSL scabs binding to dDHL-BSA was determined using a BIAcore 2000 (BIAcore, Sweden). A CM5 chip was activated with 0.2 M EDC [1-3-(3-dimethyl-aminopropyl)carbodiimide-HCl] / 0.05 M NHS (*N*-hydroxy-succinimide), and dDHL-BSA or BSA alone coupled to the chip in 10 nM Na-acetate at pH 3.5 or 4.5 respectively. A series of 10 concentrations of scAb (100 to 1000 nM)

were assayed in duplicate in HBS buffer at a flow rate of 20 microlitres/min. Between samples the chip was regenerated with 20 microlitres 100 mM NaOH. Kinetics were determined using the BIAevaluation 3 software package (Table 3).

5      The ability of the scAb G3B12 to bind to OHHL was further assessed by immobilising scAb to nickel-sepharose beads in a column via a 6 x histidine tag, and passing a solution of OHHL through the column. Any OHHL bound by the scAb and retained on the column was subsequently eluted. The concentration of OHHL in the column flow though (i.e. unbound) and that bound and later eluted were determined.

10     The ability of the scAbs to bind to HSLs and to modulate the response of bacteria to AHLs was determined using *E. coli* strains JM107 containing the plasmid pSB401 (*Vibrio fischeri* response surrogate) and JM109 containing the plasmids pSB406 and pSB406 (*Pseudomonas aeruginosa* response surrogate). The reporter plasmids contain the HSL response regulator genes *luxR* (pSB401), *lasR* (pSB1075, responsive to long-chain HSLs) or *rhlR* (pSB406, responsive to short-chain HSLs), and the *luxI* promoter region, which together with exogenous HSLs activates expression of the *luxCDABE* gene fusion (the luminescence structural genes) from *Photorhabdus luminescens*. Under the appropriate growth conditions these cells are induced to emit light in response to the presence of extra-cellular HSLs, the intensity of light emitted being proportional to the concentration of HSL.

25     Soluble scAbs from clones selected from the library were expressed using published protocols (Strachan et al., 1998). During immobilised metal affinity chromatography purification (IMAC), scAb was not eluted from the nickel-sepharose column. A series of additional scAbs with specificities to irrelevant antigens were also expressed and immobilised onto nickel-sepharose columns to act as controls. Five hundred microlitres of 10 nM OHHL was applied to each column and incubated for 1 hour at 4°C. Columns were centrifuged at 40 g for 15 s and the flow through collected. Any bound OHHL was eluted with 250 microlitres 1 M NaCl. The original flow through was re-applied and incubated as before, the flow through collected and bound HSL eluted with 1 M NaCl.

Samples of HSL solution prior to and after passage through the immobilised scAb column were applied to *E. coli* JM107 pSB401 cultures and the light emitted measured with a luminometer. Appropriate control experiments were carried out  
5 using a column to which no scAb had been immobilised, and three additional columns including scAb with specificity's for irrelevant antigens. Cells were grown shaking at 37°C for 18 h in LB medium containing tetracycline. One millilitre of the culture was inoculated into 100 ml LB tetracycline medium and grown at 37°C until an OD 600 nm 0.2 was achieved. One hundred microlitres of the culture was applied to replicate wells of a 96-well black bio-assay plate, and an equal volume of HSL solution added.  
10 HSL solutions were 10 nM OHHL (positive control), milli-Q water passed through a nickel-sepharose column (resin control), or the flow through from passing 10 nM OHHL over columns containing immobilised scAb as described above. Plates were incubated at 37°C for 2 h with shaking, and luminescence read using an Anthos LUCY1 luminometer for 1 s (Figure 4).

The ability of the scAbs to reduce bacterial responses to long-chain HSLs was assessed with an HSL-inducible luminescence reporter bioassay over a period of 3.0 h using *E. coli* strain JM109-pSB1075. This strain is essentially as described for  
20 JM107-pSB401, the difference being that plasmid pSB1075 includes the *lasR* of *Pseudomonas aeruginosa* in place of the *luxR* of *Vibrio fischeri*. Single colonies of JM109-pSB1075 were inoculated into 10 ml LB broth with antibiotic and incubated overnight at 37°C. Two hundred microlitres of overnight culture were inoculated into 10 ml fresh medium and incubated at 37°C with shaking to OD 600 nm 0.2. HSL  
25 was added to the cultures (dDHL-COOH at 20 nM final conc'n or tDHL at 50 nM final conc'n) and one hundred microlitres of culture was added to triplicate wells of a black 96 well plate. LB medium was added to negative controls. Either 50 microlitres PBS or 50 microlitres scAb at 2 mg/ml was added to each well and the plate incubated further for three hours shaking at 37°C, after which time  
30 luminescence was measured at 30 min intervals and the effect of scAb on cell signalling determined (Figures 5 and 6). The data demonstrates the ability of anti-

HSL antibodies to cross react with structurally different homoserine lactone signal molecules, and to reduce or eliminate the response of a *Ps. aeruginosa* surrogate to extra-cellular HSL.

- 5      The ability of the scAbs to reduce bacterial responses to short-chain HSLs was assessed in a similar way to that described above. The bioluminescence reporter system used *E. coli* strain JM109 with the reporter plasmid pSB406, including the *rhlR* response element regulator. The signal molecule BHL (as acyl-HSL in Fig 1 but 4 carbon side chain) was added to the *E. coli* cultures to 50 nM final concentration (an 10     equivalent volume of LB medium was added to negative control cultures), and 100 µl culture added to triplicate wells of the assay plate. Either 50 µl scAb at 100 nM or 50 µl PBS was then added, and plates incubated as described earlier. Measurements of luminescence were taken after 60 min and 150 min (Figure 7).

15     **Example 2**

- To assess the ability of anti-HSL scabs to afford protection to animals against pathogenic *Ps. aeruginosa*, a ‘slow-killing’ assay using the nematode *C. elegans* was employed. This assay is based on the killing of the worms following establishment of 20     a *Ps. aeruginosa* infection in the animal’s gut.

*Ps. aeruginosa* strain PA14 was infected into 5 ml LB broth on day 1 and incubated overnight at 37°C. On day 2, 1% of the overnight culture was inoculated into 5 ml fresh LB with 100 µl scAb at 100 nM broth and incubated at 37°C to OD 600 nm of 25     0.4. Ten microlitres bacterial culture was spotted onto the centre of NG enriched peptone agar plates (nematode growth media), together with 50 µl scAb at 120 nM and plates incubated overnight at 37°C. On day 3 (pm) an additional 50 µl scAb (120 nM) was spotted onto the plates and incubation continued overnight. On day 4 (am) the plates were transferred to room temperature (~20°C) and a further 50 µl scAb 30     added. On day 5 (am), 50 µl scAb was added as before, and 20-5- adult worms added directly onto the bacterial lawn (time = 0 h). Supplementary additions of scAb were

made at time = 26, 50 and 76 h. The numbers of dead worms were determined at intervals over the following 3 days (Figure 8a). Worms were considered dead when non-motile and not responsive to touch by a fine wire pick.

- 5 For control plates either PBS or an irrelevant scAb (specific for an unrelated target antigen) were used, or worms were grown on *E. coli* strain OP50.

A second assay was also carried out using *Ps. aeruginosa* strain PA01 (Darby *et al.*, 1999). This strain is used primarily for 'paralytic killing' (toxin production), but it  
10 also suitable, though less effective than PA14, for slow killing infection studies as in this example.

The assay was carried out as described above with the following modifications. Additions of scAb throughout were at 100 nM concentration. On day 3, 50 µl scAb  
15 was spotted onto plates in the morning and afternoon. After the second addition, the plates were transferred to room temperature and incubated overnight. Worms and scAb were applied on day 4 (time = 0 h), and only two supplementary scAb additions made at t= 30 and 60 h (Figure 8b).

20 **Example 3**

*Ps. aeruginosa* produces several extracellular products that, after colonisation, can cause extensive tissue damage. One of these, elastase, is essential for maximum virulence of *Ps. aeruginosa* during acute infection. The production of elastase is under  
25 the control of the lasI/R quorum-sensing cascade. The detection of elastase production can therefore be used as an indicator of the capacity for virulence of the bacterial population.

Four microlitres *Ps. aeruginosa* strain PA14 at 10<sup>sup</sup> 5 CFU per ml (low OD) or 10<sup>sup</sup> 8 CFU per ml (high OD) were inoculated onto agar plates containing 1% elastin, 0.5% lab lemco powder, 1% peptone, 0.5% sodium chloride and 1.5% agar, and

incubated at 37°C for 5 days. Growth to low OD discourages pathogenic switching, whereas growth to high OD encourages pathogenic switching. Fifty microlitres of scAb (200 nM) was added to the plates together with the bacteria, and additional applications of the same volume made at 24 h intervals throughout the assay.

5

The diameter of the bacterial colonies and the surrounding clear zones (indicative of lysis of elastin by elastase) were measured daily, and the elastolytic activity of the colonies determined as a ratio of the clear zone area to bacterial colony area. Again, 3-4 replicates per trial were performed and *E. coli* XL1-Blue was used as a negative control (Table 5).

10

#### Example 4

In order to isolate anti-HSL antibodies with higher affinity for antigen, and to direct specificity towards particular HSL variants, affinity maturation was performed on clone G3B12. Phagemid DNA was isolated from the G3B12 bacterial clone, and the variable light chain gene amplified by PCR using a 5' oligonucleotide primer, LINKER-REV comprising the last 30 bases of the 45 base-pair flexible linker region (5'-GGCGGAGGTGGCTCTGGCGGTAGTGC-3') and a 3' primer gIII-FOR (5'-GAATTTCTGTATGAGG-3'), specific for the phage minor coat protein gene gIII. Product of the correct size (~380 bp) was electrophoresed in a 1% agarose gel, excised, and purified. In a similar way, phagemid DNA containing the entire human naïve library from which the original clone was isolated was prepared. The whole repertoire of variable heavy chain genes was amplified using the 5' primer AH1-REV (5'-AAATACCTATTGCCTACGGC-3') specific for the pelB leader sequence, and the 3' primer LINKER-FOR encoding the first 30 bases of the linker region (5'-AGAGCCACCTCCGCCTGAACGCCCTCCACC-3'). Product of the correct size (~400 bp) was purified as above. The repertoire of VH genes was then combined with the monoclonal VL gene by linking PCR using the complementary 15 bases of the centre of the linker region that was common to both primary PCR products. The new library was amplified by addition of the primers AH1-REV and gIII-FOR to the linking PCR reaction after 4 cycles, and a further 25 cycles performed. The amplified

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DNA was digested with the restriction enzymes *NcoI* and *NotI*, and ligated into a similarly digested and purified phagemid vector. Ligated and re-purified DNA was finally transformed into *E. coli* strain TG1 cells by electroporation and plated in the conventional manner.

5

Phage antibodies were rescued with helper phage as before, and applied to immunotubes coated with dDHL-BSA conjugate, and allowed to bind. Unbound phage were poured off, and weak / non-specific binders removed by multiple wash steps with PBST and PBS. Conjugate specific phage were then eluted with low pH 10 triethylamine and neutralised. These were infected into fresh log-phage TG1 cells, rescued, and amplified again for following rounds of selection (pans). For the successive pans, the immobilised conjugate was alternated between dDHL-BSA and dDHL-TG. During the third round of panning, the bound phage from the shuffled library were eluted from duplicate pans with either free dDHL or with BHL 15 (butyrylhomoserine lactone).

After 3 rounds of panning, monoclonal phage-antibodies were screened for desired binding characteristics. Individual phage clones from round 3 were screened by ELISA: Each clone was assayed initially for the ability to bind to each of the dDHL-BSA and KLH conjugates and to the carrier proteins alone. Those clones able to bind both conjugates but unable to bind either carrier protein were further assayed to identify those whose binding to conjugate could be inhibited by the presence of free dDHL or HSL in solution. The antibody variable region genes from those phage clones found to bind to free BHL/dDHL were sub-cloned into a soluble expression 20 vector (pIMS 147), and produced as soluble single-chain antibody fragments (scAb) comprising the variable heavy and light chain domains joined by a flexible peptide linker, and a kappa constant domain from a human antibody. Quantification of the binding of soluble scAb to free HSLs was determined by competitive inhibition 25 ELISA. Samples containing a constant concentration of each selected scAb (with respect to 1 microgram per ml dDHL-TG) were incubated with a range of concentrations of free HSL for 1 h, then applied to an ELISA plate coated with tDHL-TG. After 1 h incubation, unbound scAb was washed off and any scAb remaining 30

bound to the immobilised conjugate detected with enzyme-labelled anti-human kappa antibody. The sensitivity of scAb for free HSL, and cross reactivity with other HSLs was determined from the concentration of free antigen that reduced the binding of scAb (without free antigen) to dDHL-TG by 50% (IC<sub>50</sub>) (Table 4).

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#### Example 5

Two conventional peptides, YST-1 (YSTGGAGSGG) and YST-2 YSTASGGASS were synthesised, together with a third version, YST-3, with biotinylation<sup>^</sup> (^ denotes site of biotinylation) of the penultimate C-terminal lysine side chain (YSTAGGSGAK<sup>^S</sup>). A fourth thiolactone peptide YSTC\*DFIM\* (Agr-D1) was also synthesised, where \* denotes residues connected by the thiolactone ring (see Figure 1c). Some of YST-1 was conjugated to BSA and YST-2 to bovine thyroglobulin (TG) via the C-terminus using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) by conventional conjugation chemistry.

- A human naïve antibody library was panned for four rounds against alternating immobilised YST-1 and YST-2 conjugates essentially as described previously. After binding phage-antibodies for 1 h, unbound phage were poured away and weakly bound phage removed by extensive washing with PBST followed by PBS. Bound phage were eluted with triethylamine and neutralised, then infected into log phase *E. coli* TG1 cells. The enriched phage were amplified by rescuing with helper phage, then purified and concentrated by polyethylene glycol precipitation ready for the next round of selection. Following washing of phage bound to YST-1/2-conjugates in rounds 3 and 4, phage were eluted with a solution of YST-3. Those phage binding to the biotinylated peptide were captured by streptavidin coated paramagnetic beads and immobilised with a magnet. After further wash steps, the bound phage were added directly to TG1 Cells and allowed to infect as before.
- Following four rounds of selection, monoclonal phage-antibodies were screened by ELISA for binding to YST-1 and YST-2 conjugates, and to BSA and TG carrier proteins alone. The scFv genes of those clones that bound only to both conjugates

were sub-cloned into the pIMS-147 scAb soluble expression vector and transformed into *E. coli* XL1-Blue cells. These cells were expressed, and soluble scAb extracted from the bacterial periplasmic space and purified by Nickel affinity chromatography. Purified scAb was then further assayed for binding to free (non-conjugated) peptide  
5 and to the thiolactone-peptide autoinducer Agr-D1, in competition with immobilised peptide conjugates by ELISA. Signal reduction in the presence of peptide compared to binding to conjugate alone indicates that scAb is recognising the YST-epitope common to all peptides (Figure 9).

10 **Example 6**

In order to generate antibodies against the AI-2 target, both the free AI-2 molecule and a conjugated form are required. It is not (considered) possible to isolate AI-2 in a pure form. In nature, AI-2 is formed by the (spontaneous) reaction of pro-AI-2  
15 (figure 1(b)) with boric acid. *In vitro*, pro-AI-2 will also react with boric acid to yield active AI-2, however this is not suitable for conjugation and antibody selection. A derivative of pro-AI-2 can be synthesised whereby the methyl group is replaced by a linker e.g. an acyl chain, with a terminal reactive group e.g. carboxylic acid. Any structure that includes a terminal reactive group suitable for chemical conjugation or  
20 cross-linking to a carrier, and that will result in the core pro-AI-2 moiety being displayed clear of the carrier surface, can be used as a linker. The reactive pro-AI-2 is conjugated to preferably two different carriers as described in the section 'summary of the invention'.

25 A library of potential receptors (e.g. an antibody library displayed on phage) is applied to an immobilised conjugate in the presence of boric acid (preferably > 10 µM, pH 6.0-8.0) in order to yield immobilised AI-2 conjugate. Phage-antibodies ('phage') are allowed to bind, and those not recognising conjugate are removed by washing. Bound phage can be eluted with high or low pH e.g. triethylamine, or by  
30 competitive binding with free AI-2 or by competitive binding with e.g. biotinylated AI-2 followed by removal with magnetic streptavidin beads. During all stages of bio-panning except extreme pH elution, borate should be present to ensure that the correct

structure of AI-2 is maintained. Eluted phage should be re-infected into host bacteria (*E. coli*), amplified by growing cells under phage-particle producing conditions and purified for the next round. Subsequent rounds of selection are carried out as described for round one except that, preferably, the immobilised conjugate is  
5 alternated.

- When sufficient rounds of selection have been completed, individual (monoclonal) clones can be assayed for binders to AI-2. It is probable that at least three rounds of selection will be needed, although AI-2 binding clones may be isolated after only one  
10 round, or it may be necessary to perform more than three rounds. Polyclonal phage-ELISA can be performed after each round by methods well known to those familiar with the art to determine how many rounds are required. Monoclonal phage-antibodies should be produced as described in earlier examples, and assayed for binding to each of the conjugates used for selection and to the respective unconjugated carriers. Third or fourth conjugate(s) may be used additionally if available.  
15 Putative positive clones will be identified as those binding to all available conjugates in the presence of borate, but not to carrier molecules alone and preferably not to conjugate in the absence of borate.
- 20 As it is possible that the reaction of pro-AI-2 with borate may yield more than one species, it will be preferable to demonstrate that antibodies recognise in particular the correct AI-2 structure. This could be determined by assaying for binding to conjugate in the presence of free pro-AI-2 and borate. A reduction in binding with increasing concentrations of free pro-AI-2 is indicative of competitive inhibition. Such  
25 antibodies would therefore be expected to be able to modulate the response of AI-2 responsive bacteria by binding to extracellular AI-2 and rendering it unavailable to cells.

A suitable *in vivo* model can be found in bioluminescent *Vibrio harveyi*, a bacterium  
30 that bioluminesces in response to AI-2. Strains of *V. harveyi* that are LuxS<sup>-</sup> are unable to synthesise DPD (a precursor of pro-AI-2), and so cannot produce AI-2. They are however able to respond by (increased light output) in response to

exogenously added AI-2, either in the form of pro-AI-2 together with borate, or as borate-containing cell-free culture media obtained from LuxS<sup>+</sup> *V. harveyi*. The addition of borate alone to LuxS<sup>-</sup> cells or to LuxP<sup>-</sup> cells (lacking the natural AI-2 receptor) does not result in any light emission. Potential anti-AI-2 antibodies ('receptors') could therefore be identified as those fulfilling the binding criteria outlined earlier, and also being able to either deplete AI-2 from w.t. *V. harveyi* culture media as determined by reduced light emission when added to LuxS<sup>-</sup> cells, or to quench/prevent/reduce light emission when added to LuxS<sup>+</sup> cells.

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**BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE**

Haptogen Ltd  
Polworth Building  
Foresterhill  
Aberdeen  
AB25 2ZD

**INTERNATIONAL FORM**

**RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITORY AUTHORITY  
identified at the bottom of this page**

**NAME AND ADDRESS  
OF DEPOSITOR**

**I. IDENTIFICATION OF THE MICROORGANISM**

Identification reference given by the  
DEPOSITOR:

*Escherichia coli*  
G3B12

Accession number given by the  
INTERNATIONAL DEPOSITORY AUTHORITY:

NCIMB 41168

**II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

The microorganism identified under I above was accompanied by:

a scientific description

a proposed taxonomic designation

(Mark with a cross where applicable)

**III. RECEIPT AND ACCEPTANCE**

This International Depository Authority accepts the microorganism identified under I above, which was received by it on  
18 March 2003 (date of the original deposit)<sup>1</sup>

**IV. RECEIPT OF REQUEST FOR CONVERSION**

The microorganism identified under I above was received by this International Depository Authority on  
(date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it  
on (date of receipt of request for conversion)

**V. INTERNATIONAL DEPOSITORY AUTHORITY**

Name: NCIMB Ltd.,

Signature(s) of person(s) having the power to represent the  
International Depository Authority or of authorised  
official(s):

Date: 27 March 2003

Address: 23 St Machar Drive,  
Aberdeen,  
AB24 3RY,  
Scotland.

<sup>1</sup> Where Rule 6/4(d) applies, such date is the date on which the status of International Depository Authority was acquired.

Applicant's or agent's file reference number	P35262WO/NCB	International application No.
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**INDICATIONS RELATING TO A DEPOSITED MICROORGANISM**

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>17</u> , line <u>17</u> .	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;"><input checked="" type="checkbox"/> Further deposits are identified on an additional sheet</span>	
Name of depositary institution NCIMB Ltd	
Address of depositary institution ( <i>including postal code and country</i> ) 23 St Machar Drive Aberdeen AB24 3RY Scotland	
Date of deposit  <u>18 March 2003</u>	Accession Number  <u>NCIMB 41169</u>
C. ADDITIONAL INDICATIONS ( <i>leave blank if not applicable</i> ) <span style="float: right;"><input type="checkbox"/> This information is continued on an additional sheet</span>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ( <i>if the indications are not for all designated States</i> )  In respect of all designated States to which such action is possible and to the extent that it is legally permissible under the law of the designated State, it is requested that a sample of the deposited biological material be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), UK Patent Rules 1995, Schedule 2, Paragraph 3, Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated State.	
E. SEPARATE FURNISHING OF INDICATIONS ( <i>leave blank if not applicable</i> )  The indications listed below will be submitted to the International Bureau later ( <i>specify the general nature of the indications e.g. "Accession Number of Deposit"</i> )	

For receiving Office use only

This sheet was received with the international application

*[Signature]*

Authorized officer

For International Bureau use only

This sheet was received by the International Bureau on:

Authorized officer

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INTERNATIONAL DEPOSITORY AUTHORITY  
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**NAME AND ADDRESS  
OF DEPOSITOR**

**I. IDENTIFICATION OF THE MICROORGANISM**

Identification reference given by the  
DEPOSITOR:

*Escherichia coli*  
G3G2

Accession number given by the  
INTERNATIONAL DEPOSITORY AUTHORITY:

NCIMB 41169

**II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

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 a proposed taxonomic designation

(Mark with a cross where applicable)

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official(s).

Address: 23 St Machar Drive,  
Aberdeen,  
AB24 3RY,  
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Date: 27 March 2003

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Applicant's or agent's file reference number	P35262WO/NCB	International application No.
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

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B. IDENTIFICATION OF DEPOSIT	
Name of depositary institution NCIMB Ltd	
Address of depositary institution ( <i>including postal code and country</i> ) 23 St Machar Drive Aberdeen AB24 3RY Scotland	
Date of deposit <u>18 March 2003</u>	Accession Number <u>NCIMB 41170</u>
C. ADDITIONAL INDICATIONS ( <i>leave blank if not applicable</i> ) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ( <i>if the indications are not for all designated States</i> )  In respect of all designated States to which such action is possible and to the extent that it is legally permissible under the law of the designated State, it is requested that a sample of the deposited biological material be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), UK Patent Rules 1995, Schedule 2, Paragraph 3, Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated State.	
E. SEPARATE FURNISHING OF INDICATIONS ( <i>leave blank if not applicable</i> )  The indications listed below will be submitted to the International Bureau later ( <i>specify the general nature of the indications e.g., "Accession Number of Deposit"</i> )	
For receiving Office use only <input checked="" type="checkbox"/> This sheet was received with the international application <i>Newman</i>	
For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on:	
Authorized officer	
Authorized officer	

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RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
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**NAME AND ADDRESS  
OF DEPOSITOR**

**I. IDENTIFICATION OF THE MICROORGANISM**

Identification reference given by the  
DEPOSITOR:

*Escherichia coli*  
G3H3

Accession number given by the  
INTERNATIONAL DEPOSITORY AUTHORITY:

NCIMB 41170

**II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

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 a proposed taxonomic designation

(Mark with a cross where applicable)

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Signature(s) of person(s) having the power to represent the  
International Depository Authority or of authorised  
official(s):

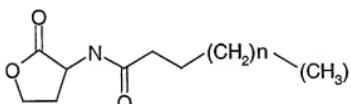
Address: 23 St Machar Drive,  
Aberdeen,  
AB24 3RY,  
Scotland.

Date: 27 March 2003

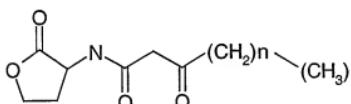
<sup>1</sup> Where Rule 6/4(d) applies, such date is the date on which the status of International Depository Authority was acquired.

CLAIMS

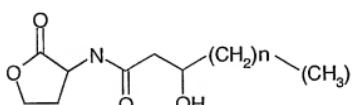
1. An antibody to a lactone or a lactone-derived signal molecule secreted by  
5 bacteria.
2. An antibody as claimed in claim 1, in which the lactone signal molecule is a homoserine molecule or a peptide thiolactone molecule.
- 10 3. An antibody as claimed in claim 2, in which the homoserine lactone molecule has a general formula selected from the group consisting of:



Formula I



Formula II



Formula III

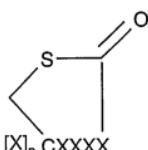
15 where n = 0 to 12..

4. An antibody as claimed in claim 3, in which the homoserine lactone molecule of general formula I is N-butanoyl-L-homoserine lactone (BHL) where n = 0, N-dodecanoyl-L-homoserine lactone (dDHL) where n = 8 and n-tetradecanoyl-L-homoserine lactone (tDHL) where n = 10.
- 20

5. An antibody as claimed in claim 3, in which the homoserine lactone molecule of general formula II is *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) where n = 2 and *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) where n = 8.

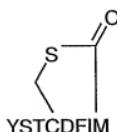
10 6. An antibody as claimed in claim 3, in which the homoserine lactone molecule of general formula III is *N*-(3-hydroxybutanoyl)-L-homoserine lactone (HBHL) where n = 0.

7. An antibody as claimed in claim 2 in which the peptide thiolactone has a general formula (IV) as follows:

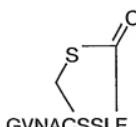


where X is any amino acid and n = 1 to 10.

15 8. An antibody as claimed in claim 7, in which the peptide thiolactone molecule is:

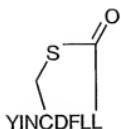


or



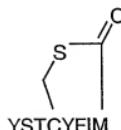
or

5



10

or



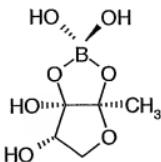
15

9. An antibody as claimed in claim 1, in which the lactone-derived signal molecule is a furanosyl borate diester.

20

10. An antibody as claimed in claim 9, in which the furanosyl borate diester is Auto Inducer-2 (AI-2),

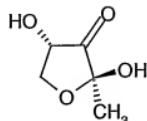
30



35

11. An antibody as claimed in claim 1, in which the lactone-derived signal molecule is Pro-AI-2 or a C<sub>1</sub>-C<sub>10</sub> saturated or unsaturated carboxylic acid derivative thereof

5



15 12. An antibody as claimed in any one of claims 1 to 11 which is a polyclonal antibody

13. An antibody as claimed in any one of claims 1 to 11 which is a monoclonal antibody.

20

14. An antibody as claimed in any one of claims 1 to 11 which is a single chain antibody (scAb)

25

15. An antibody as claimed in any one of claims 1 to 11 which is an antibody fragment.

16. An antibody as claimed in claim 15, in which the antibody fragment is a single chain variable fragment (scFv).

30

17. An antibody as claimed in claim 15, in which the antibody fragment is a single domain fragment.

18. A pharmaceutical composition comprising an antibody as defined in any one of claims 1 to 17.

35

19. A method for the treatment of bacterial infection of a subject, the method comprising administration of an antibody as defined in any one of claims 1 to 17.

20. A kit of parts comprising an antibody as defined in any of claims 1 to 17 provided in unit dosage form.
- 5 21. An antibody as defined in any one of claims 1 to 17 for use in medicine.
22. The use of an antibody as defined in any one of claims 1 to 17 for use in the preparation of a medicament for the treatment of bacterial infection.
- 10 23. A method of screening a population of specific binding molecules for an anti-bacterial specific binding molecule, the method comprising conjugating a bacterial lactone or lactone-derived signal molecule to a carrier molecule and using the conjugate so formed to identify a specific binding molecule that specifically binds to the conjugate from the population of specific binding molecules.
- 15 24. A method as claimed in claim 23, in which the specific binding molecule is an antibody or a fragment thereof.
25. A method as claimed in claim 24, in which the antibody is a monoclonal antibody.
- 20 26. A method as claimed in claim 24, in which the antibody is a polyclonal antibody.
- 25 27. A method as claimed in any one of claims 23 to 26, in which the carrier molecule is a protein.
28. A method as claimed in any one of claims 23 to 27, in which the bacterial lactone signal molecule is a homoserine molecule or a peptide thiolactone molecule.

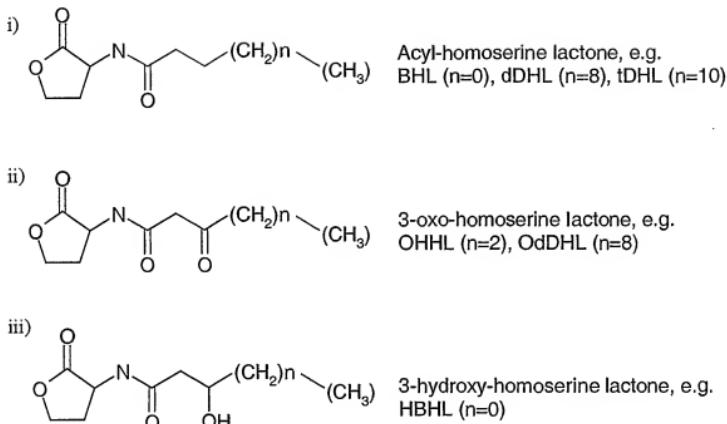
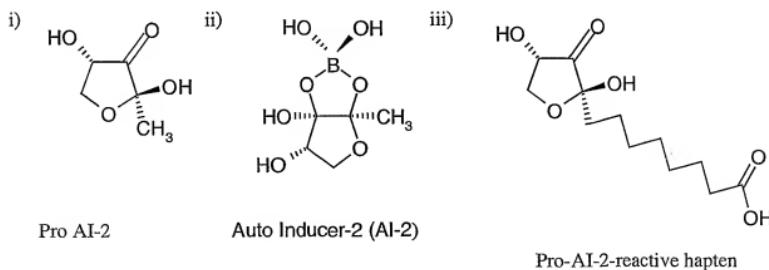
29. A method as claimed in any one of claims 23 to 27, in which the lactone derived signal molecule is a furanosyl borate diester, such as AI-2, or Pro-AI-2 or a C<sub>1</sub>-C<sub>10</sub> saturated or unsaturated carboxylic acid derivative thereof.
- 5      30. A method as claimed in any one of claims 23 to 29, in which the population of specific binding molecules is a phage display library.
- 10     31. A specific binding molecule identified by a method of any one of claims 23 to 30 for use in medicine.
- 15     32. The use of a specific binding molecule identified by a method according to any one of claims 23 to 30 in the preparation of a medicament for the treatment of a bacterial infection.
- 20     33. The use of a bacterial lactone or lactone-derived signal molecule to screen a population of specific binding molecules in order to identify a specific binding molecule that specifically binds to said bacterial lactone signal molecule.
- 25     34. A method of treatment of a bacterial infection of a subject, the method comprising isolation of a bacterial lactone or lactone-derived signal molecule in a sample from said subject and using said bacterial lactone signal molecule to screen a population of specific binding molecules for an anti-bacterial specific binding molecule to identify a specific binding molecule that specifically binds to the signal molecule, and administering said specific binding molecule so identified to a patient in need thereof.
- 30     35. A method as claimed in claim 34, in which the sample is of blood, saliva, tissue, cerebro-spinal fluid, tears, semen, urine, faeces, pus, skin, or mucous secretions.

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Table 1

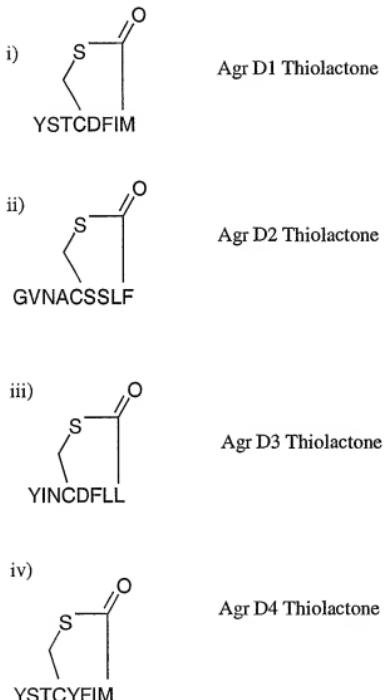
Organism	Regulatory proteins	Signal molecule	Phenotype
<i>Aeromonas hydrophila</i>	AhyI/AhyR	BHL, HHL	serine protease, metalloprotease
<i>Aeromonas salmonicida</i>	Asal/AsaR	BHL, HHL	?
<i>Agrobacterium tumefaciens</i>	TraI/TraR	HHL, OOHL	conjugation
<i>Burkholderia cepacia</i>	CepI/CepR	OHL	ornibactin, siderophores, exoprotease
<i>Chromobacterium violaceum</i>	CviI/CviR	HHL	antibiotics, violacein, exoenzymes, cyanide
<i>Erwinia carotovora</i>	CarI/CarR Expl/ExplR	OHHL +?	carbapenem antibiotic exoenzymes
<i>Erwinia chrysanthemi</i>	Expl/ExplR	OHHL, HHL	pectate lyase
<i>Escherichia coli</i>	?/SdiR LuxS/?	?	cell division
<i>Pantoea stewartii</i>	EsaI/EsaR	OHHL	Exopolysaccharide
<i>Pseudomonas aeruginosa</i>	LasI/LasR RhII/RhIIR	OdDHL, OHHL, OOHL BHL, HHL	Virulence factors inc: alkaline protease, elastase exotoxin A Chitinase, pyocyanin, rhamnolipid
<i>Pseudomonas aureofaciens</i>	PhzI/PhzR	BHL, HHL	Phenazine antibiotic
<i>Pseudomonas fluorescens</i>	PhzI/PhzR	OHL, HHHL, HOHL, HDHL	Phenazine antibiotic
<i>Ralstonia solanacearum</i>	SolI/SolR	HHL, OHL	? (aidA)
<i>Rhizobium leguminosarum</i>	RhlI/RhlR	HHL, OHL	Nodulation
<i>Rhodobacter sphaeroides</i>	CerI/CerR	?	Community escape EPS expression
<i>Serratia liquefaciens</i>	SwrI/?	BHL, HHL	Swarming, phospholipase
<i>Vibrio anguillarum</i>	VanI/VanR	ODHL	?
<i>Vibrio fischeri</i>	LuxI/LuxR AinS/AinR	OHHL, HHL OHL	Bioluminescence
<i>Vibrio harveyi</i>	LuxI/LuxR LuxPQ/LuxS	HBHL AI-2	bioluminescence
<i>Xenorhabdus nematophilus</i>	?	HBHL	Virulence factors
<i>Yersinia enterocolitica</i>	YenI/YenR	HHL, OHHL	?
<i>Yersinia pestis</i>	YpeI/YeR	HHL, OHHL	Pathogenicity
<i>Yersinia pseudotuberculosis</i>	YpsI/YpsR YtbI/YtbR	HHL, OHHL OHL	Flagella production ?
<i>Basillus anthracis</i>	?	AI-2	Virulence factors

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**Figure 1a.****Figure 1b.**

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Figure 1c.



**Table 2.**

scAb	dDHL-COOH	tDHL	OHHL	dDHL-BSA	Paraquat
G3G2	11 µM	21 µM	17 mM	0.28 µM	N/D
G3B12	4 µM	5 µM	2 mM	0.32 µM	N/D

N/D indicates that no IC<sub>50</sub> value could be determined.

**Table 3.**

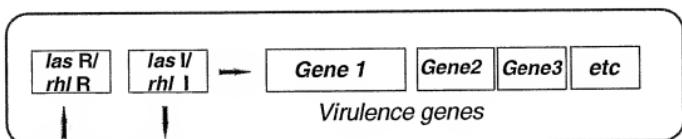
scAb	ka (mol l <sup>-1</sup> s <sup>-1</sup> )	kd (s <sup>-1</sup> )	KA (mol/l)	KD (nM)
G3G2	4.19 x 10 <sup>4</sup>	1.43 x 10 <sup>-3</sup>	2.93 x 10 <sup>7</sup>	34.1
G3B12	3.93 x 10 <sup>4</sup>	1.56 x 10 <sup>-3</sup>	2.52 x 10 <sup>7</sup>	39.7

**Table 4.**

scAb	BHL	dDHL	tDHL	OHHL	Paraquat
G3B12	17 mM	1.4 mM	4.5 mM	10 mM	N/D
L1-A7 (G3B12)	1.25 mM (× 13.6)	40 µM (× 35)	-	-	N/D
L1-B7 (G3B12)	-	130 µM (× 10.8)	-	-	N/D
L1-C11 (G3B12)	-	200 µM (× 7)	-	-	N/D
G12 (G3B12)	3 mM (× 5.7)	-	0.6 mM (× 7.5)	5 mM (× 2)	N/D

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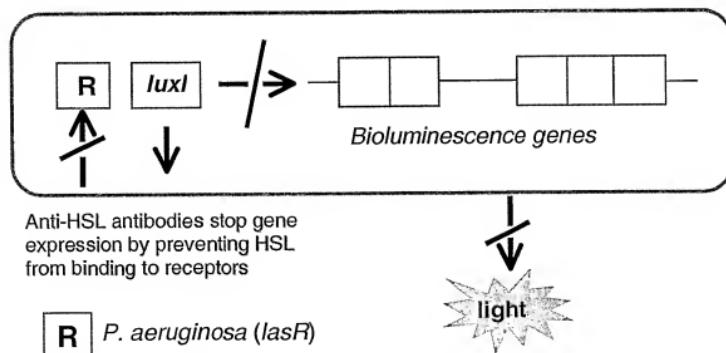
Figure 2.

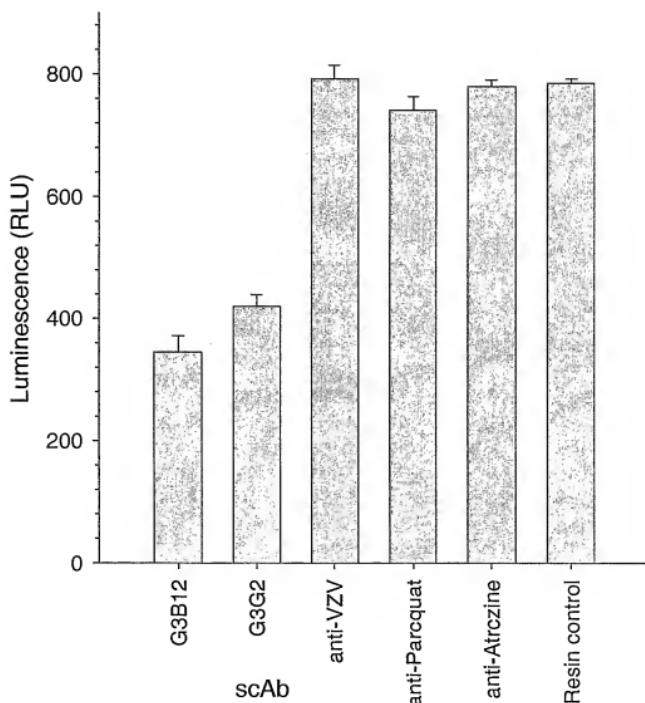
**regulation**

***las I*/  
*rhl I*** Produce long chain (*las I*) and short chain (*rhl I*) HSLs

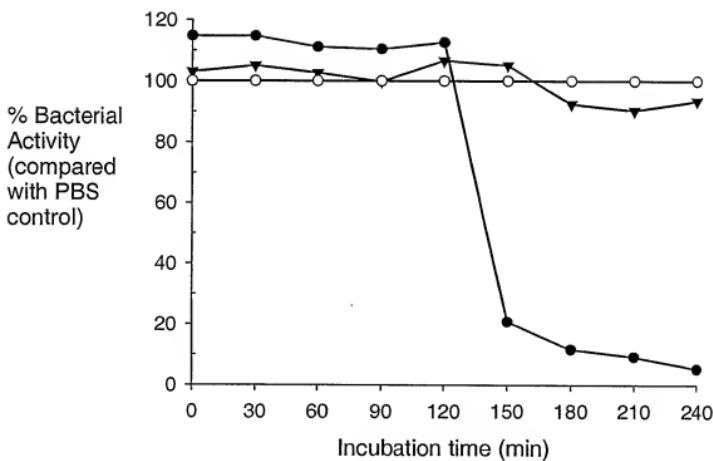
***las R*/  
*rhl R*** Respond to long chain (*las R*) and short chain (*rhl R*) HSLs

Figure 3.



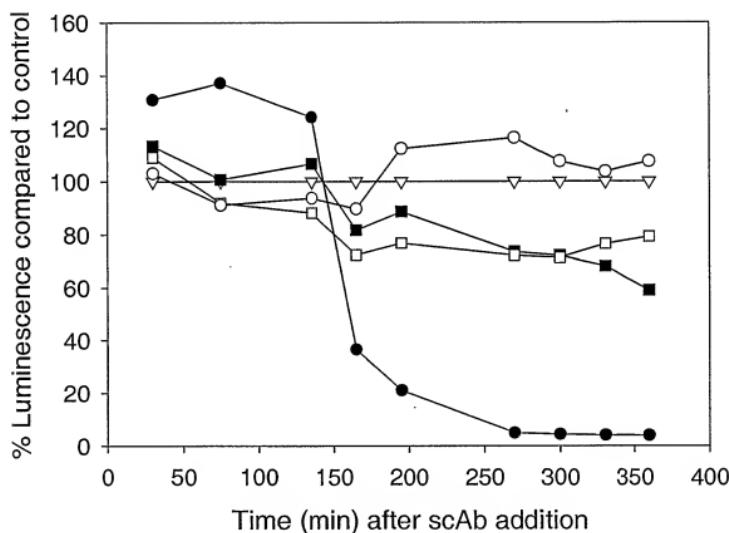
**Figure 4.**

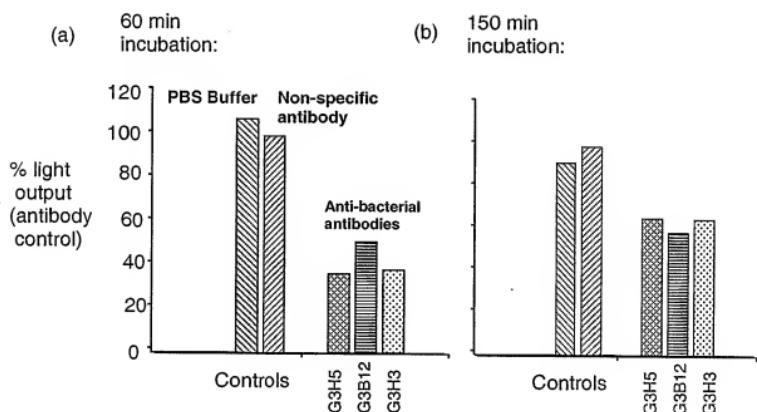
7 / 12

**Figure 5.**

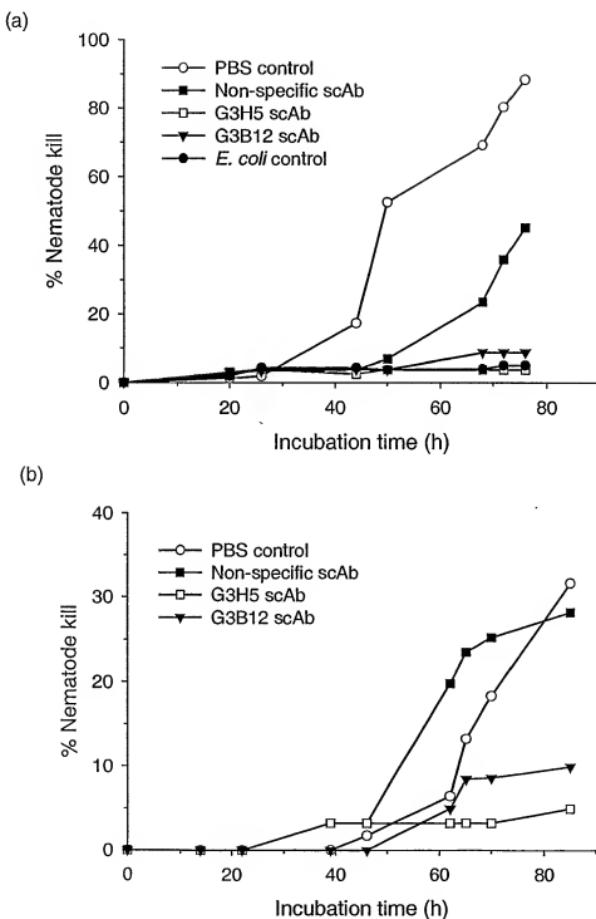
8/12

Figure 6.



**Figure 7.**

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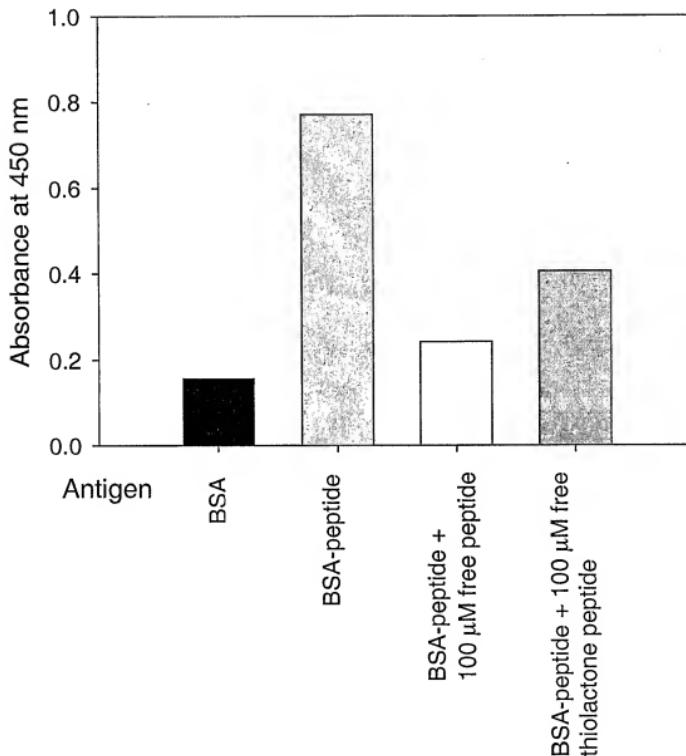
**Figure 8.**

**11/12****Table 5.**

High Optical density						Low density	
Inc Days	PBS Control	Control scAb	G3H5	G3B12	G3H3	PBS Control	3 scAb mixture
1	100	100	100	100	100	100	100
2	100	100	100	100	100	100	82
3	100	95	70	68	84	100	75
4	100	100	58	52	65	100	65
5	100	95	52	47	49	100	56

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Figure 9.



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 03/03529

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 A61K39/395 A61K39/40 A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 94543 A (QUAY STEVEN C ;QUAY ENTPR LLC K (US)) 13 December 2001 (2001-12-13) page 3, line 16-20 page 5, line 14 -page 6, line 9 page 27, line 20-26 claims 39,52-61,64-67,71,72,74,75,81-83,85,88-98, 105 --- -/- -	1-35

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the International filing date
- \*U\* document which may throw doubt on priority; claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but which understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*Z\* document member of the same patent family

Date of the actual completion of the International search

Date of mailing of the International search report

28 November 2003

12/12/2003

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COVONE-VAN HEES, M

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB 03/03529

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WU H ET AL: "Detection of N-acylhomoserine lactones in lung tissues of mice infected with <i>Pseudomonas aeruginosa</i>." <i>MICROBIOLOGY</i> (READING, ENGLAND) ENGLAND OCT 2000, vol. 146 ( Pt 10), October 2000 (2000-10), pages 2481-2493, XP002263253 ISSN: 1350-0872 abstract page 2482, left-hand column, paragraph 3 page 2491, right-hand column, paragraph 3 -page 2492, left-hand column, paragraph 1</p>	1-35
X	<p>REVERCHON SYLVIE ET AL: "New synthetic analogues of N-acyl homoserine lactones as agonists or antagonists of transcriptional regulators involved in bacterial quorum sensing." <i>BIOORGANIC &amp; MEDICINAL CHEMISTRY LETTERS</i>. ENGLAND 22 APR 2002, vol. 12, no. 8, 22 April 2002 (2002-04-22), pages 1153-1157, XP002263254 ISSN: 0960-894X introduction</p>	1-35
A	<p>WO 99 27786 A (LYNCH MARTIN JOHN ;SWIFT SIMON (GB); WILLIAMS PAUL (GB); FISH LEIG) 10 June 1999 (1999-06-10) page 2, line 7-9 page 4, paragraph 3 -page 5, paragraph 1 page 7</p>	1-35
A	<p>DONG Y-H ET AL: "QUENCHING QUORUM-SENSING-DEPENDENT BACTERIAL INFECTION BY AN N-ACYL HOMOSERINE LACTONASE" <i>NATURE</i>, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 408, no. 6839, 14 June 2001 (2001-06-14), pages 813-817, XP001093866 ISSN: 0028-0836 the whole document</p>	1-35
A	<p>HARTMAN G ET AL: "Quorum sensing: potential means of treating gram-negative infections?" <i>LANCET</i>. ENGLAND 21 MAR 1998, vol. 351, no. 9106, 21 March 1998 (1998-03-21), pages 848-849, XP001160986 ISSN: 0140-6736 the whole document</p>	1-35
-/-		

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB 03/03529

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FINCH R G ET AL: "Quorum sensing: a novel target for anti-infective therapy." THE JOURNAL OF ANTIMICROBIAL CHEMOTHERAPY. ENGLAND NOV 1998, vol. 42, no. 5, November 1998 (1998-11), pages 569-571, XP002263255 ISSN: 0305-7453 the whole document -----	1-35

## INTERNATIONAL SEARCH REPORT

Interr. application No.  
PCT/GB 03/03529

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  

Although claims 19,34,35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No  
PCT/GB 03/03529

Patent document cited in search report	Publication date		Patent family member(s)		Publication date
WO 0194543	A 13-12-2001	AU EP WO	7499801 A 1299118 A2 0194543 A2		17-12-2001 09-04-2003 13-12-2001
WO 9927786	A 10-06-1999	AU WO	1252499 A 9927786 A1		16-06-1999 10-06-1999